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Review

The PAS-domain kinase PASKIN: a new sensor in energy homeostasis

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Keywords. Diabetes mellitus, glucose tolerance, glycogen synthesis, insulin, metabolic syndrome, nitrogen fixation, protein translation, respiration.

Introduction

Energy homeostasis is central to life. All organisms need to adapt to nutrition availability by reducing energy expenditure when food is scarce and by storing energy when food is plentiful. Whereas many important insights have been obtained into the signalling pathways and effector mechanisms involved in metabolic energy adaptation, less is known about the actual cellular mechanisms 'sensing' primary nutritional compounds and secondary metabolic intermediates. This review focuses on PASKIN, a novel protein that contains a possibly sensory PAS domain and an effector Ser/Thr kinase domain. Recent results suggest that PASKIN serves as a sensor involved in metabolic energy homeostasis.

The PAS domain: heterodimerization interface and ligand binding site

The ~7000 PAS folds identified up to date maintain similar structures despite low sequence conservation and a large functional flexibility. The name 'PAS' is an acronym derived from the three PAS fold-containing transcription factors period (PER), aryl hydrocarbon receptor nuclear translocator (ARNT) and single-minded (SIM). PER is a component of a network of

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transcription factors, all of them containing PAS domains, which compose the circadian clock [1]. ARNT is the common heterodimerization partner of the aryl hydrocarbon receptor (AHR), also called dioxin receptor, and the hypoxia-inducible factor (HIF) α subunits [2, 3]. SIM controls midline cell specification during neurogenesis, and *SIM* is a critical locus gene in Down syndrome [4–6].

As part of transcription factors, the PAS region usually consists of two adjacent degenerate repeats of ~130 amino acids, called PAS A and PAS B. PAS domains contain five antiparallel β-sheets flanked by α-helices (Fig. 1). In basic-helix-loop-helix (bHLH) DNA-binding proteins, the main role of the PAS region is to confer specific protein-protein interactions within the family (Fig. 1a). However, the three-dimensional structure of the PAS domain resembles a left-handed glove, and just like a baseball in a mitt, the PAS structure is also well-suited to host small-molecule ligands (Fig. 1b). Indeed, AHR was discovered based on its role in the mammalian xenobiotic response, and AHR is the only known biological receptor that binds the environmental pollutant dioxin. Neuronal NPAS2, a circadian clock component and a putative tumour suppressor, is another mammalian PAS transcription factor reported to contain a ligand. Each PAS domain of NPAS2 coordinates a heme group that specifically binds carbon monoxide [7]. Both the possible endogenous ligand of AHR and the physiological meaning of the carbon monoxide binding by NPAS2 remain under investigation. Up to date, no other mammalian transcription factors have been reported to contain ligand-binding PAS domains.

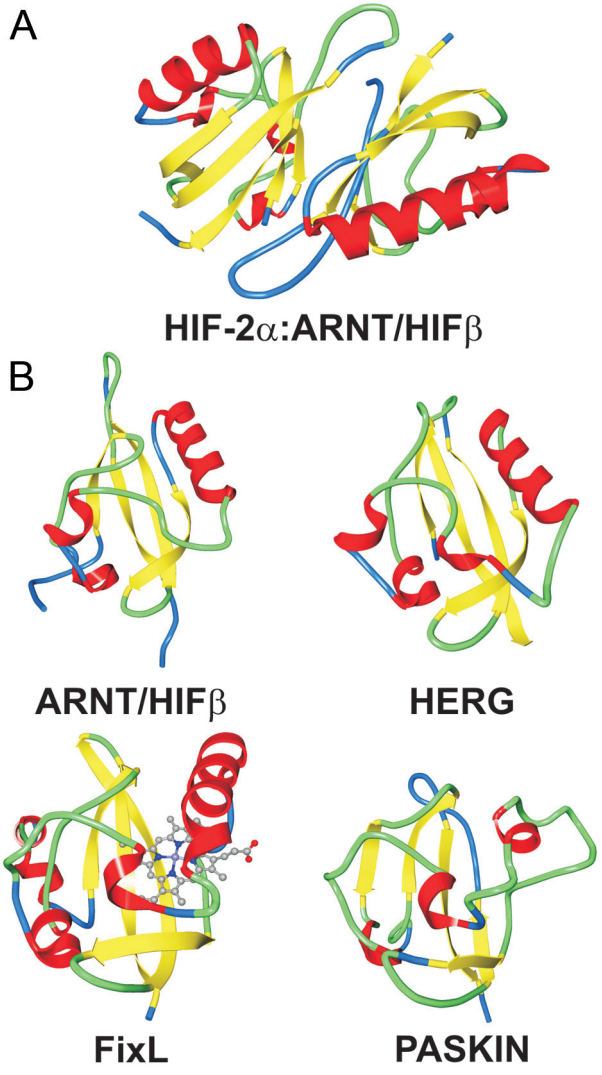

Figure 1. Structures of various PAS domains. (*A*) PAS domain heterodimerization between HIF-2α and ARNT/HIFβ. (*B*) Structural similarity between various PAS domains. Structures were prepared using Sirius1.2 software (http://sirius.sdsc.edu/) based on the following Protein Data Bank coordinates: HADDOCK structure of HIF-2α:ARNT/HIFβ heterodimer (2A24) [35]; NMR structure of ARNT/HIFβ (1X0O) [35] and PASKIN (1LL8) [20]; X-ray structure of HERG (1BYW) [12] and FixL including the heme group (1D06) [36]. ARNT, aryl hydrocarbon nuclear translocator; HERG, human *ether-à-go-go*-related gene; HIF, hypoxia-inducible factor.

The archaeal and bacterial ancestors of mammalian PAS proteins

Physiological adaptations of an organism to changing environmental conditions require molecular sensors capable of sensing and signalling specific physicochemical parameters. Especially in Bacteria and Archaea, the PAS domain is often found in environmental protein sensors involved in the perception of external signals, including light intensity, gas partial pressures, redox potentials, and certain organic ligands [8–11]. In the mammalian HERG potassium channel, the PAS domain might even sense membrane potentials [12].

Bacterial *Rhizobium* and *Bradyrhizobium* species live in root nodules of some plants, such as soybean and clover. Oxygen sensing in these species is required for regulated expression of the oxygen-sensitive enzymes involved in nitrogen fixation. The PAS domain in the histidine kinase FixL serves as a heme-bearing molecular oxygen sensor. Upon oxygen binding by the ferrous iron within the heme group, the PAS domain changes its conformation and inhibits the histidine kinase domain, regulating the activity of the FixJ transcription factor in an oxygen-dependent manner [13]. Other bacterial gas-responsive PAS proteins include the *Escherichia coli* direct oxygen sensor (*Ec*Dos) and the *Burkholderia xenovorans* regulator of CO metabolism (RcoM). Similar to eukaryotic

NPAS2, bacterial RcoM coordinates heme and binds carbon monoxide and nitric oxide [14].

PASKIN, a mammalian relative of bacterial oxygen sensors

Of physiological interest, the oxygen-regulated HIF transcription factors contain PAS domains just like the oxygen sensing FixL of nitrogen-fixing bacteria. In analogy to the binding of dioxin and carbon monoxide by the PAS domains of AHR and NPAS2, respectively, this raised the question as to whether the HIF PAS domain can sense oxygen directly. However, no evidence was found for HIF being a direct oxygen sensor. Moreover, in the meantime it became clear that oxygen sensitivity is conferred by oxygen-dependent protein hydroxylation of HIF α subunits, leading to transcriptional inactivation as well as polyubiquitination and proteasomal destruction [3, 15]. Nevertheless, this intriguing relation between the bacterial and mammalian oxygen-sensing systems raised the possibility that a mammalian homolog of the bacterial oxygen sensors might be involved in oxygen sensing in mammals. Only a single mammalian gene could be found which has a domain architecture similar to FixL, i.e. a PAS region combined with a kinase domain. We called the human and mouse genes *PASKIN* and *Paskin*, respectively [16]. Interestingly, the nucleotide sequence provides more evidence that *PASKIN* might have evolved from the FixL gene. First, the PASKIN PAS region shares a higher sequence similarity with the FixL PAS region than with any other known PAS domain. Second, a single long exon between the PAS B and serine/threonine kinase regions of *PASKIN* is weakly similar to the histidine kinase domain of FixL and only few point mutations within the introns interrupt an even longer open reading frame, suggesting that the histidine kinase domain might have been replaced by a serine/threonine kinase domain during evolution [16]. However, the presence of potential sensing and signalling domains raised the possibility that PASKIN might serve as a sensor protein.

The PAS domain of PASKIN inhibits serine/threonine kinase domain-dependent autophosphorylation

Despite the structural relation, we found heme bound to FixL but not to PASKIN (unpublished data), and there is ample evidence to rule out a role for PASKIN in direct oxygen-sensing in mammals. A first hint for another kind of functional relationship between FixL and PASKIN was provided by the group of S. L. McKnight, J. Rutter and colleagues who cloned human PASKIN and alternatively termed it PAS kinase or PASK [17]. However, since this name had been in use before for an unrelated kinase [18, 19], we herein refer to the unambiguous designation 'PASKIN' throughout this review. McKnight, Rutter and colleagues reported that PASKIN autophosphorylates the threonine residues Thr1161 and Thr1165 and that the N-terminal PAS A domain of PASKIN partially represses the C-terminal kinase activity *in trans* as well as *in cis*. Following de-repression, presumably by ligand binding to the PAS domain, autophosphorylation *in trans* results in the 'switch-on' of the kinase activity of PASKIN. Mutations of Thr1161or Thr1165 completely inactivated PASKIN kinase activity [17].

Further evidence for intrinsic kinase inhibition by the PAS domain was provided by structural studies: in contrast to other PAS proteins, the PAS A domain of PASKIN contains a short F α-helix and a long and dynamic FG loop that interacts with the kinase domain. Distinct mutations mimicking the ligand-bound state de-repressed the kinase activity without affecting the overall structure of the PAS A domain [20]. Thus, it was tempting to speculate that PASKIN binds (so far unknown) organic ligands, increases its kinase activity by autophosphorylation and signals to (so far unknown) downstream phosphoproteins.

PASKIN has also been found in a large biochemical screen for nuclear phosphoproteins in HeLa cells, confirming that PASKIN is a phosphoprotein and providing a hint for its subcellular localization [21]. In this study, PASKIN was found to be phosphorylated at Ser116. Since Ser116 is located adjacent to the PAS A domain, it potentially might regulate the PAS domain function. However, to date neither the kinase nor the functional implications of this phosphosite are known.

PSK1 and PSK2, the yeast homologs of PASKIN

Important insights into the function of PASKIN were derived from the cloning and analysis of two genes with high sequence similarity from *Saccharomyces cerevisiae*, termed PSK1 and PSK2 [22]. These budding yeast homologs of PASKIN phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, suggesting that they might coordinately control translation and sugar flux. Under specific stress conditions (a temperature-sensitive growth defect on galactose medium, called Gal^{ts} phenotype), albeit not under standard culture conditions, yeast glycogen synthase and UDP-glucose pyrophosphorylase (Ugp1) are

phosphorylated by Psk1 and Psk2, resulting in the downregulation of carbohydrate storage, whereas deficiency in Psk1 and Psk2 resulted in elevated glycogen stores. Simultaneously, Psk2-dependent phosphorylation of the yeast translation factors Caf20p (corresponding to 4E-BP in mammals), eIF1A and Sro9p leads to the downregulation of protein synthesis [22]. Further experiments revealed that the Gal^{ts} phenotype might be exclusively related to the lack of Ugp1 Ser11 phosphorylation in the PSK mutant yeast strains. Upon Ser11 phosphorylation, Ugp1 translocates to the plasma membrane where it increases cell wall glucan synthesis at the expense of glycogen storage. In the absence of PSKs, glycogen rather than glucan is produced, affecting the strength of the cell wall. This effect was fully phenocopied by a Ugp1 Ser11Ala mutation, and it could be reversed by adding the osmotic stabilizer sorbitol [23]. Two independent cell stressors have been identified to activate PSKs in yeast. Cell integrity stress (e. g. heat shock or SDS treatment) required the Wsc1 membrane stress sensor, and growth in non-glucose carbon sources (e. g. raffinose) required the AMP-dependent kinase (AMPK) homolog Snf1 [24]. While PSK2 was predominantly activated by Wsc1, PSK1 was indispensable for Snf1 function.

Because the Ser/Thr kinase domain of PASKIN is highly related to the kinase domains of AMPK family members (30 and 32 % identity over a stretch of 253 amino acids for AMPKα1 and AMPKα2, respectively), it is tempting to transfer this latter finding to the mammalian system. However, despite its relationship with bacterial FixL, PASKIN most probably does not serve as a mammalian oxygen sensor; likewise, a putative role for PASKIN as an AMPK-like energy sensor in mammalian cells needs to be approached with caution. Indeed, the PASKIN Ser/Thr kinase domain is also highly similar to the *PIM1*, *PIM2* and *PIM3* oncogenes (35, 37 and 33 % identity, respectively) and generally shares 28–37 % sequence identity with a number of other kinases of the calcium/calmodulin-dependent protein kinase family. Thus, functional predictions based only on sequence comparisons with the kinase domain are likely to be of minor relevance. Regarding the second function of PSKs in yeast, mammalian Ugp1 is not a PASKIN target, and the yeast Ugp1 Ser11 phosphorylation site is not conserved in mammalian Ugp1 [25].

Parallels between yeast and mammalian PASKIN downstream targets

The exciting findings regarding the function of the PASKIN homologs in yeast raised the question as to whether similar downstream targets phosphorylated by PASKIN might exist in mammalian cells. Indeed, the mammalian muscle glycogen synthase has subsequently been reported to be a PASKIN target [26]. Glycogen synthase is known to be regulated by a number of kinases targeting various phosphorylation sites. PASKIN inactivates glycogen synthase by phosphorylation at Ser640. The middle region of PASKIN between the PAS and kinase domains interacts with glycogen synthase. Glycogen inhibits this interaction, suggesting a glycogen-sensing function [26].

By yeast two-hybrid screening for novel protein interactors of PASKIN, we found that the eukaryotic translational elongation factor eEF1A1 is phosphorylated by PASKIN at Thr432, probably leading to increased translation elongation, at least following PASKIN overexpression [27]. Additional screenings of kinase target peptide arrays revealed novel potential targets of PASKIN that are known to be involved in the regulation of sugar flux and protein synthesis, including the ribosomal subunit S6, which is also phosphorylated by S6-kinase, a major regulator of protein translation (unpublished data). However, neither the biological stimulus nor the physiological relevance of PASKIN-dependent phosphorylation of glycogen synthase and translation factors is currently known. Thus, additional work will be required to clarify the functional relation between yeast PSK and mammalian PASKIN function.

Is the PASKIN PAS A domain binding a ligand?

The 3-dimensional structure of the PASKIN PAS A domain has been resolved, and synthetic ligands binding to this domain were identified [20]. The identified molecules are slightly reminiscent of the dioxin structure with two aromatic rings as most prominent feature. Intriguingly, ligand binding as well as ligand-mimicking mutations relieved the kinase domain from PAS domain inhibition, as is known from FixL following oxygen binding to the heme-bearing PAS domain. However, these synthetic ligands bind only with rather low affinity, and no data have been provided to date for putative cellular functions of the synthetic ligands. Thus, despite the beauty of the concept of ligand-mediated kinase de-repression, it is important to note that neither an endogenous ligand of PASKIN has been identified nor has this concept been confirmed in a cellular system, including yeast, or in animals *in vivo*.

Generation and initial characterization of *Paskin* knock-out mice

In order to better understand the physiological role of PASKIN in mammals, we targeted the mouse *Paskin* gene by homologous recombination in embryonic stem cells. Therefore, exons 10–14, forming the upstream and N-terminal parts of the kinase domain, were replaced by an IRES/*lac*Z reporter gene [28]. This loss-of-function mutation maintained normal expression of a truncated PASKIN mRNA and hooked it up to a β-galactosidase reporter. It cannot be excluded that the truncated PASKIN N-terminal region might have some biological effects, but since no suitable antibodies are available, the expression levels of this truncated protein remain unknown. However, while the PAS domain is still present, at least on the mRNA level, a potential truncated PASKIN protein will be non-functional because central regions and the kinase domain are missing.

Initial analyses revealed that PASKIN mRNA expression in wild-type mice and β-galactosidase mRNA in heterozygous animals was identical, allowing the identification of PASKIN expressing cell types by *in situ* X-gal staining for β-galactosidase activity. Surprisingly, PASKIN expression is strongly upregulated in post-meiotic germ cells during spermatogenesis [28, 29]. In fact, PASKIN mRNA in testis is at least 100-fold higher than in any other organ examined. Assuming that physiological functions and the major expression site of PASKIN will overlap, we concentrated our initial investigations on the testis. However, neither male fertility nor sperm production or motility were affected in homozygous knock-out mice. The animals do not show any gross alterations. In particular, life expectancy, food intake, energy expenditure, locomotor activity (unpublished data kindly provided by P. Wielinga, B. Alder, C. Loewenstein and T. Lutz, Zurich, Switzerland), fat distribution and liver fat content (unpublished data kindly provided by J. Hillebrand and W. Langhans, Zurich, Switzerland) and circadian activity under constant dark conditions (unpublished data kindly provided by I. Tobler, Zurich, Switzerland) are indistinguishable from control littermates. Despite glycogen synthase being a downstream target of PASKIN, periodic acid-Schiff staining did not reveal any alterations in glycogen deposits in the tissues of *Paskin* knock-out mice (unpublished data). Thus, at least under standard animal husbandry conditions *Paskin* knock-out mice do not display any developmental, morphological or behavioural phenotype.

A recent report suggested that PASKIN might be involved in respiratory regulation [30]. Female but not male *Paskin* knock-out mice showed an increased ventilatory response to acute hypoxia and failed to reach ventilatory acclimatization to chronic hypoxia. L-DOPA production as a means to determine tyrosine hydroxylase activity was decreased in catecholaminergic cells of the brainstem of male but increased in female *Paskin* knock-out mice when compared to wild-type animals. Peripheral chemoreceptors were not affected in *Paskin* knock-out mice, consistent with a lack of PASKIN expression in carotid bodies (unpublished data kindly provided by J. López-Barneo, Sevilla, Spain). Of note, body temperature, O_2 consumption and CO_2 production decreased following exposure to hypoxia, but the values were indistinguishable between *Paskin* wild-type and knock-out mice of either sex [30]. Despite its putative significance considering the bacterial ancestors of PASKIN, these studies failed to provide a mechanistic explanation for the respiratory differences. Especially the relation between tissue-specific PASKIN expression, tyrosine hydroxylase activity and the oxygenation- and sex-specific functions deserve further investigations.

A role for PASKIN in glucose-stimulated insulin production in the pancreas?

Intriguingly, it has been suggested that PASKIN kinase activity followed by mRNA and protein expression is increased in MIN6 cells and in isolated pancreatic β-cells after exposure to high glucose concentrations [31]. Increased PASKIN activity appeared to be required for glucose-dependent transcriptional induction of the pancreatic duodenal homeobox-1 (PDX-1) transcription factor, leading to transcriptional induction of preproinsulin but not glucokinase or uncoupling protein 2 gene expression. Mutations of the PDX-1 DNA binding site on both the preproinsulin promoter and the *PDX1* promoter itself inhibited glucose and PASKIN overexpression-dependent promoter activity [31]. In contrast to this *PDX1* gene-activating function of PASKIN, it has subsequently been reported by the same group that PASKIN phosphorylates PDX-1 at Thr152, which might drive PDX-1 out of the nucleus [32]. However, the authors concluded that 'decreases in PASK activity in β cells may thus contribute to some forms of type 2 diabetes, whereas activation of the enzyme may provide a new therapeutic strategy for this disease' [31].

To examine the role of PASKIN in glucose-stimulated insulin production, we used pancreatic β-cells derived from our *Paskin* wild-type and knock-out mice [33]. We found PASKIN mRNA regulation by high glucose neither in various pancreatic β-cell or testicular cell lines, nor in isolated islets or tubuli seminiferi. Increasing the ambient glucose concentration in

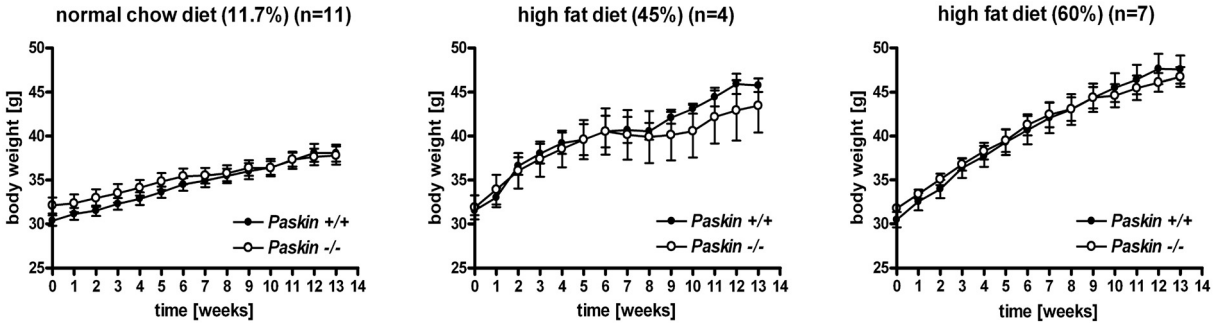

Figure 2. Body weight gain of wild-type and *Paskin* knock-out mice fed with increasingly fat-containing diets (11.7, 45 and 60 % calories by fat, respectively). Male mice received the indicated diets beginning at the age of 14 weeks. Mean values ± SEM of the indicated number of mice per group are shown.

cultured islets resulted in a similar increase in insulin mRNA and insulin secretion, whether the β-cells were derived from *Paskin* wild-type or knock-out mice. In young as well as in older *Paskin* wild-type and knock-out mice, glucose tolerance tests showed equal blood glucose clearance, whether the animals were fed a normal chow diet or fasted overnight. These experiments demonstrated normal acute and chronic insulin function in *Paskin* knock-out mice *in vivo*, leading to the conclusion that insulin expression is independent of PASKIN, at least under our laboratory conditions [33]. A lack of constitutive and glucose-induced PASKIN protein expression was confirmed in human β-cells (unpublished data kindly provided by M. Donath, Zurich, Switzerland).

Paskin knock-out mice seem to be protected from all aspects of the metabolic syndrome when fed a high-fat diet

Excitingly, when feeding mice with a high fat diet (HFD, 45 % fat by calories), J. Rutter and colleagues found that *Paskin* knock-out mice are protected from obesity, liver triglyceride accumulation, glucose intolerance and peripheral insulin resistance, the symptoms common to the metabolic syndrome [34]. These differences were accounted for by an increase in energy turnover as measured by O_2 consumption and CO_2 production. The molecular mechanisms leading to this interesting phenotype are unclear. An involvement of mitochondrial biogenesis, AMPK and mTOR activity could be excluded. As in skeletal muscle of *Paskin* knock-out mice, a partial (~50 %) PASKIN knock-down in L6 myoblasts resulted in increased glucose and fatty acid oxidation with a concomitant increase in ATP levels [34]. While these results implied a cell-autonomous effect of PASKIN on energy turnover, we did not find any differences in ATP levels in mouse embryonic fibroblasts derived from wild-type or *Paskin* knock-out mice (unpublished data), suggesting that these effects are cell type-specific. Considering the phenotype of *Paskin* knock-out mice (see above), constitutive changes in intracellular ATP production are also unlikely to occur in β-cells of the pancreas.

The lean phenotype of *Paskin* knock-out mice appeared only after feeding a HFD. Under normal chow diet (NCD), body weight, fat composition, body temperature, O_2 consumption and CO_2 production of *Paskin* knock-out mice are not distinguishable from wild-type littermates [30, 34]. After an independent repetition of the feeding studies, we could confirm that an NCD made no significant difference, whereas a HFD led to a stronger increase in body weight in wild-type mice than in *Paskin* knock-out mice (unpublished data). Rather surprisingly, when we further increased the fat content of the food (from 45 to 60 % calories by fat) there was no longer any difference in body weight increase between wild-type and *Paskin* knock-out mice (Fig. 2). Currently, we have no explanation for this unexpected finding.

It is important to mention that the same *Paskin* knock-out mouse strain was used for these experiments as for all other studies mentioned above [28]. The only notable difference was that the mice were backcrossed into C57BL/6 five times in the Hao et al. study [34], whereas we used the 10th backcross. Despite an apparently impaired glucose-stimulated insulin secretion by islets of Langerhans isolated from *Paskin* knock-out mice, glucose and insulin tolerance were not significantly different between wild-type and knock-out mice fed an NCD [34]. Moreover, in contrast to the previous report [31], PDX-1 mRNA levels were independent of PASKIN in this study [34]. While these apparently contradictory data are difficult to interpret, they confirm our previous results [33].

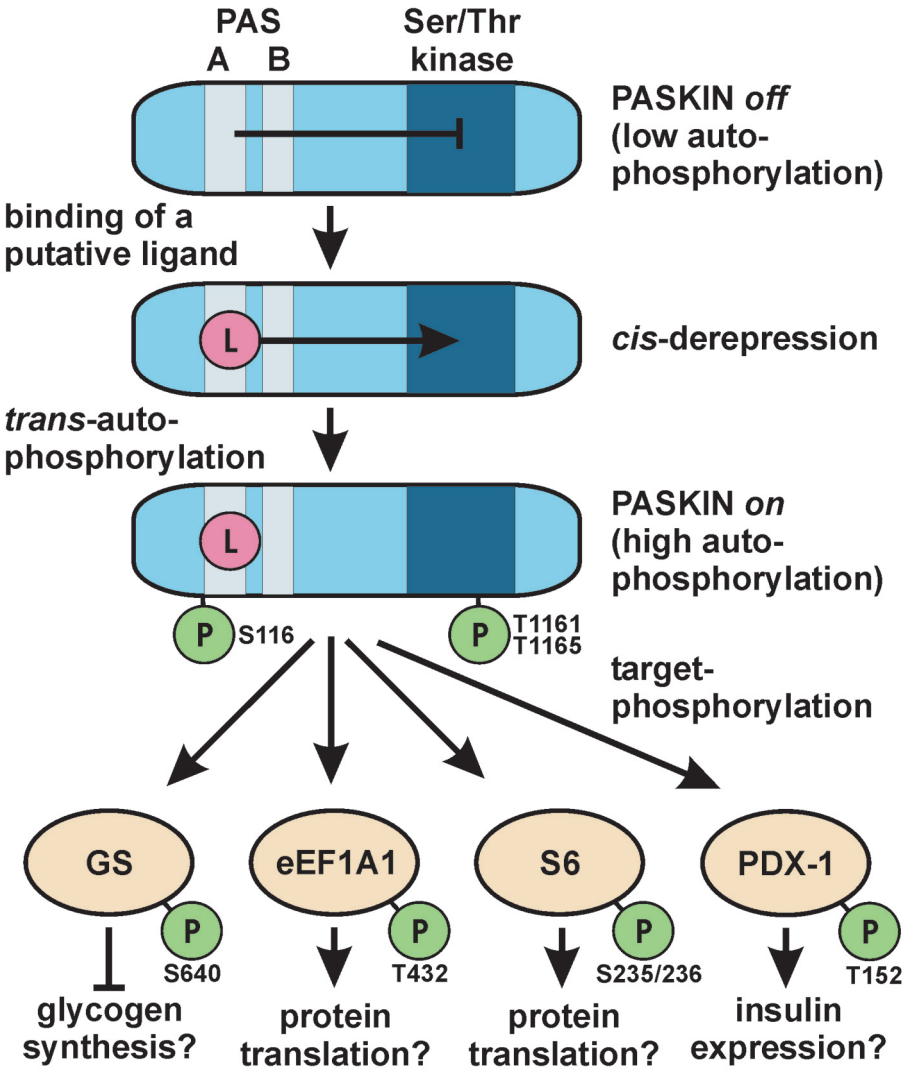

Figure 3. Model of PASKIN-dependent signalling. Upon binding of an unknown organic ligand, PASKIN autophosphorylation and target phosphorylation increase, resulting in the regulation of critical factors in energy homeostasis. GS, glycogen synthase; eEF1A1, eukaryotic translation elongation factor 1A1; PDX-1, pancreatic duodenal homeobox-1.

Conclusions

Figure 3 provides an overview of the current model of the molecular functions of PASKIN as a potential sensor protein. Much less is known about the physiological significance of PASKIN. Similar to yeast, the reports on the functions of mammalian PASKIN clearly demonstrate that PASKIN plays a role under changing environmental conditions (oxygen, glucose, nutrition) rather than under standard laboratory conditions. This might also explain the different outcomes of similar experiments in different laboratories: subtle changes in nutritional and/or other environmental parameters of the animals as well as of the cell cultures might have influenced PASKIN-dependent cellular effects. However, in contrast to the previous suggestion [31], it appears to be the decrease rather than an increase in PASKIN activity that protects from the metabolic syndrome, at least in the mouse model described in this review. PASKIN has been suggested to provide a signal for 'metabolic sufficiency' by sensing a yet unknown metabolic ligand [25, 34]. Pharmaceutical inhibition of PASKIN thus might be useful for treatment of the metabolic syndrome, especially regarding the finding that PASKIN deficiency apparently does not affect the health of knock-out mice. It will be a major challenge to identify the endogenous ligand of PASKIN. Only knowledge of the nature of this ligand will eventually clarify the role of PASKIN as an energy sensor.

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